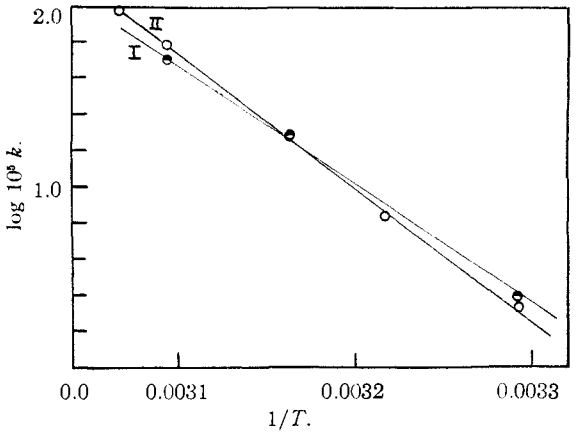

Fig. 1.—Plots for calculation of activation energies for the alkaline dehydrochlorination of β-benzene hexachloride in 81.5 (I) and 92.6 (II) weight % ethanol.

cross. This has been pointed out previously,[13,14] but seems to be frequently overlooked.

Our data (Tables II, IV and VI) indicate that, in the limited systems studied, increasing ethanol does increase the rate constants at 10° for α-BHC and at 30° for ε-BHC, but that this rate increase is caused by favorable entropy effects at these temperatures, whereas the energy of activation is apparently increased by increasing ethanol. The β-isomer actually gives a maximum in rate constant, which again is an entropy effect and is opposed by the effect of activation energy, which also shows a maximum. The direct or inverse relationships between rate constants and Arrhenius activation energies, are, as mentioned above, functions of the temperatures at which the data were obtained and thus have no simple theoretical significance.

We are unable to account for the appearance of the maxima for the β-isomer and the lack of such maxima with the α-isomer. Similar maxima have been observed previously in water–organic solvent mixtures.[15–19]

(13) D. Pressman and W. G. Young, *ibid.*, **66**, 705 (1944).

(14) A. G. Evans and S. D. Hamann, *Trans. Faraday Soc.*, **47**, 40 (1951).

(15) M. Prasad and R. D. Godbole, *J. Ind. Chem. Soc.*, **7**, 127 (1930).

(16) R. A. Fairclough and C. N. Hinshelwood, *J. Chem. Soc.*, 1573 (1937).

(17) J. E. Stevens, C. L. McCabe and J. C. Warner, THIS JOURNAL, **70**, 2449 (1948).

(18) J. W. Hackett and H. C. Thomas, *ibid.*, **72**, 4962 (1950).

(19) P. M. Nair and S. V. Anantakrishnan, *Proc. Indian Acad. Sci.*, **32A**, 187 (1950).

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The Use of the Gradient Tube for the Determination of Crystal Densities[1a]

BY BARBARA W. LOW AND F. M. RICHARDS[1b]

A study has been made of the applicability of the Gradient Column Technique to the determination of the densities of crystalline solids. The usable density range has been extended to provide coverage for a wide variety of crystalline preparations. The use of the column has led to a modification which appears better suited to the determination of solid densities. Gradient columns may be set up in 10-cc. centrifuge tubes and spun in a centrifuge without seriously affecting the gradient. This modified procedure greatly increases the speed of the density measurement. The modified gradient column is the basis of a new method for the detection and separation of impurities in crystalline preparations. The method has also been used to demonstrate the formation of mixed crystals between components of known and different densities. The use of the Gradient Column for the determination of "wet" and "dry" protein crystal densities has been investigated. Density measurements have been made on the two protein crystal preparations—β-lactoglobulin and insulin—both of which have been previously studied by other methods.

Introduction

The gradient tube was originally devised by Linderstrøm-Lang[2,3] for the determination of the specific gravity of aqueous solutions in the dilatometric micro-estimation of enzyme activity. It was later extended[4] to the determination of the densities of D_2O–H_2O mixtures in the range of density 0.99–1.01. The apparatus is capable of a sensitivity of 0.000001 g./ml. A description of the apparatus, its calibration and some modification in the procedure is given by Anfinsen.[5] The use of a simplified gradient tube prepared in a vertical measuring cylinder without thermostating was described by Jacobsen and Linderstrøm-Lang[6] for rapid though less accurate (0.1%) laboratory measurements.

Boyer, Spencer and Wiley[7] have applied the gradient tube to the study of high polymers in the three fields—crystallization rate/data, inhomogeneities in the composition of copolymers, and rates of polymerization. The density gradient column has also been used to determine the densities of natural and synthetic fibers in the study of their textural inhomogeneities.[8]

The possibility of employing the gradient tube for the determination of crystal density was suggested to us by Professor Stig Claesson.[9] We have

(1) (a) This work has been supported by the Eugene Higgins Trust, by grants from the Rockefeller Foundation, the National Institutes of Health, by contributions from industry, and by funds of Harvard University; (b) Atomic Energy Commission Predoctoral Research Fellow in the Biological Sciences.

(2) K. Linderstrøm-Lang, *Nature*, **139**, 713 (1937).

(3) K. Linderstrøm-Lang and H. Lanz, *Compt. rend. trav. lab. Carlsberg*, **21**, 315 (1938).

(4) K. Linderstrøm-Lang, O. Jacobsen and G. Johansen, *ibid.*, **23**, 17 (1941).

(5) C. Anfinsen, "Symposium Preparation and Measurement of Isotopic Tracers: The Determination of Deuterium in the Gradient Tube," J. W. Edwards, Ann Arbor, Michigan, 1948, p. 61.

(6) C. F. Jacobsen and K. Linderstrøm-Lang, *Acta Physiol. Scand.*, **2**, 149 (1940).

(7) R. F. Boyer, R. S. Spencer and R. M. Wiley, *J. Polymer Sci.*, **1**, 249 (1946).

(8) S. Tessler, N. T. Woodberry and H. Mark, *ibid.*, **1**, 437 (1946).

(9) Stig Claesson, Physical Chemistry Institute, University of Upsala, personal communication, 1946.

studied the method both for this general purpose and also with reference to the possibility of using it for the accurate determination of "wet" and "dry" protein crystal densities. The special problems associated with these latter measurements will be discussed in the appropriate section.

During the course of the study, we have investigated the use of the gradient tube for a wide range of liquid densities and with a variety of solutions. Linderstrøm-Lang[2] had earlier recognized the wide possibilities of the method.

Crystal Density and Molecular Weight Determination.—The X-ray crystallographic determination of molecular weights involves the measurement of the volume of the crystallographic unit cell (V) in Å.^{3}, the measurement of the crystal density (ρ) g./cubic milliliter and the determination of the number (n) of asymmetric units in the unit cell. The equation may be written

$$\text{M.W.(g.)} = \frac{V\,(\text{in Å.}^3) \times 10^{-24} N\rho}{n}$$

where N = Avogadro's number.

The determination of the unit cell dimensions with an accuracy varying between 0.2 and 1.0% may be easily made with the techniques presently available. A rapid technique for the measurement of crystal density to at least this order of accuracy, (when only limited amounts of small ill-formed crystals are available) is thus an important tool in the X-ray crystallographic determination of molecular weights.

In the early stages of the investigation of a new crystalline compound an accurate X-ray molecular weight determination of the asymmetric unit may be of value in establishing the correct formula for a compound where this is in some doubt.[10] Equally, it may be used to establish the presence of solvent of crystallization in the solid phase.[11]

Current Methods for the Determination of the Density of Solids.—Several authors[12,13] have discussed the standard methods of measuring solid densities. A more recent review by Mason[14] provides data for a comparison of available methods.

Of these only the Berman[15] adaptation of the Archimedes principle method for large crystals of the order of 25 mg. in weight, and the flotation method,[16] are commonly used for the type of crystals available for X-ray analysis.

For small ill-formed crystals, the flotation method is slow and tedious. Its accuracy is dependent upon the homogeneity of the sample. Because of the time required, accuracies of 0.2–1% are usually considered adequate although the method is capable of higher accuracy.[17]

(10) D. M. Crowfoot and B. W. Low, *Lancet*, 112 (1944).

(11) J. Bernal, D. Crowfoot and I. Fankuchen, *Phil. Trans. Roy. Soc.*, [A] **239**, 135 (1940).

(12) A. E. H. Tutton, "Crystallography and Practical Crystal Measurement," Vol. I, Macmillan, London, 1922, pp. 625–639.

(13) J. Reilly and W. N. Rae, "Physico-chemical Methods," 2nd Ed., New York, 1932, pp. 304–330.

(14) B. Mason, *Geol. Fören. Förh.*, **66**, 27 (1944).

(15) H. Berman, *Amer. Mineral.*, **24**, 434 (1939).

(16) J. W. Retgers, *Z. physik. Chem.*, **3**, 289 (1889).

(17) The Westphal balance is frequently used to measure the density of the liquid suspension mixtures employed. The sensitivity of the method is increased by the use of a centrifuge.[18]

(18) S. B. Hendricks and M. E. Jefferson, *J. Optical Soc. Am.*, **23**, 299 (1933).

Principle of the Gradient Tube, Its Method of Operation.—In the Linderstrøm-Lang gradient tube[2,3] a practically linear specific gravity gradient is produced by mixing kerosene and bromobenzene in varying ratios. When one liquid is layered over another of greater specific gravity, with which it is miscible, a linear gradient of density develops near the interface. Manipulation of a plunger type stirrer in a vertical tube can extend the gradient over the greater part of the column.

The process of diffusion in a column of this type is so slow that a column will maintain its gradient virtually unchanged for many months. A drop of an unknown immiscible liquid with some intermediate density introduced into the column falls until it reaches a level corresponding to its own density when it remains stationary.

Extension of the Technique.—The original column described by Linderstrøm-Lang[2] had a range of about 0.99 to 1.01. Because of the low gradient, the sensitivity was 0.0001 g./ml./mm. Hence, vibration-free mountings and careful temperature control were required ($\pm 0.002°$) to prevent convection currents. The possible range of the bromobenzene (ρ = 1.48)–kerosene (ρ = 0.79) column is about 0.8 to 1.5.

By a proper choice of liquids the density range 0.7 to 4.5 commonly found in crystalline solids may be covered. The sensitivity of the column depends primarily upon the difference in density between the two components when mixed in a column of standard length. A column covering a wide range may be used to survey the density of a series of substances. Then, to measure the density of one of these with greater accuracy, a new column may easily be prepared, expanding the density scale about this particular position. The column liquids chosen depend upon the substance being investigated and the required density range. It is, of course, essential that the substance be insoluble in and non-reacting with the column components. A column may be composed of organic components with suitable salt solutions as standards, or composed of salt solutions with suitable organic mixtures as standards. Where the insolubility requirement cannot be fulfilled the method must be used with great caution. (Alcohol-containing protein crystals are an example of this.)

Experimental

Application of the Gradient Column to the Determination of the Density of Crystalline Solids.—A few minor and experimental problems arose associated with the use of solids rather than liquid drops.

Columns were set up to investigate the general applicability of the gradient tube method to the measurement of crystal density. Most of the test crystals used were pure amino acid preparations, available to us in the laboratory. Their densities had all previously been measured largely by pycnometric methods.[19] These crystalline preparations were chosen because the crystals were small and most of them ill-formed and flaky.

(19) E. J. Cohn, T. L. McMeekin, J. T. Edsall and J. H. Weare, THIS JOURNAL, **56**, 2270 (1934).

Preparation of the Column.—The columns were prepared from bromobenzene and xylene in a 20-cm. tube. The density range covered was 1.15–1.35. No special precautions were taken to thermostat the column.

Introduction of Sample.—A solid sample put directly into the column tended simply to float on the surface. Further, when crystals are introduced into the body of a liquid they may carry air bubbles with them which would invalidate the measurements. To avoid these two difficulties a slurry of the solid and a small amount of the less dense column component was made in a test-tube. When the solid had been thoroughly covered with the liquid and all air bubbles excluded, the thin paste was introduced into the column by means of a pipet. Crystals large enough for manipulation with forceps were carefully wetted in the less dense column component before being introduced into the tube. The surface tension of aqueous solutions may be reduced by the addition of glycerol or sodium taurocholate.

Measurement of Position.—The accuracy of measurement of the position of a layer of solid or of a single large ill-formed crystal is inherently less than that for the measurement of a single drop of liquid. A cathetometer was unnecessary for tubes of this sensitivity (accuracy approximately 0.1%).[6] A millimeter rule supported alongside the tubes proved adequate for the estimation of positions which were read to 0.5 mm.

Calibration of the Gradient.—The gradient was calibrated by means of about 0.01-ml. drops of known specific gravity. Solutions of potassium phosphate with density increments of 0.002 were used. The use of drops in calibrating a column for crystalline materials has a certain disadvantage.[20] The drops may adhere to the solid material and prove difficult to remove. The possibility of calibrating the gradient by measuring the specific refractive index as a function of position was suggested to us by Dr. C. Frondel and Miss M. Morse. Two further methods of calibration by solid floats and dye distribution have been used by Tessler, *et al.*[8]

Rate of Sedimentation.—Large crystals and crystalline fragments settled as rapidly as liquid drops and the equilibrium position was attained in less than 10 minutes. Fine powders or flakes took longer (approximately 2 hours) and tended to be distributed over a considerable length of the column (*cf.* Table II). In this latter circumstance, the cut-off toward the heavy end of the tube was generally very sharp. It was composed of the smallest pieces of the sample. Air bubbles appeared to be associated with the larger fragments not completely broken up in the preliminary slurry. In these cases, the sharply layered heavy material certainly represented the true density of the solid.

Removal of Material from Column.—Most ingenious and effective methods for the removal of liquid drops have been devised for the gradient tube.[5,21] No similar method is yet available for the removal of solids from the column. Thus, after it has been used for several measurements, the usefulness of the column becomes somewhat impaired by the presence of the layers of material previously introduced.

Criticisms.—The difficulties associated with the introduction of the crystals into the tube are slight and do not affect the usefulness of the method. The time taken by finely powdered and flaky samples to settle involves some inconvenience. A more serious criticism is the necessity for replacing the column after using it for several determinations. In order to meet these criticisms a modified gradient tube method was devised.

Modified Gradient Tube

It has been found that gradient columns may be set up in 10-cc. centrifuge tubes and spun in a centrifuge without seriously affecting the gradient. The useful range of length, developed by stirring, is about 5 cm. After introducing the sample and centrifuging the entire column for 1 to 2 minutes at 2500 r.p.m., most materials layer out sharply. During 2 minutes the heating effects are negligible. In this method, the linearity of the density gradient and general uniformity of the column were not assumed. A large selection of standard solutions was kept available so that the crystal layer might be bracketed between two standard density solutions to the desired degree of accuracy after centrifugation. The amount of material involved was small so that the entire column was discarded after each measurement.[22]

Results

The results obtained are found in Table I. The crystal appearance and the character of the layering are recorded; they indicate the effect of the crystal shape and size on the accuracy of the measurements.

The densities measured, using the gradient columns, agree very closely with those obtained by other techniques. The method certainly requires less time for comparable accuracy and less material than was used in the original amino acid density studies. It may be remarked that the value for alanine obtained by us approaches as closely as the literature value to the density calculated from the unit cell dimensions.

The density of claviformin was originally measured using the flotation method. The slightly higher gradient column value ($\rho = 1.535$) leads to a somewhat better estimate (155 ± 2) of the molecular weight (156) of this compound than does the earlier observation (154 ± 3).

The distribution of material in the column discussed previously may largely be eliminated by more careful preparation of the preliminary slurry. It has been noted by various authors that organic and inorganic crystals may commonly contain small occlusions of air and mother liquor. The highest density obtained is, therefore, on this basis also, considered the most reliable.

The Detection and the Separation of Impurities in Crystalline Preparations

The method is dependent upon differences in density between preparation and contaminant(s).

The differences of density between crystalline components of a mixture have been used as a basis of a flotation method for their separation.[23] The method depends upon the choice of a liquid medium in which certain of the components sink and others float. A continuous series of solutions are used to obtain complete fractionation. The method is slow and is easily applicable only where mixtures of known approximate composition are to be fractionated into components, already characterized and of known density.

(20) One of us (F. M. R.) at the suggestion of, and in collaboration with, Mr. T. Thompson, has studied a micro Westphal balance method for the calibration of the gradient (*Anal. Chem.*, in press).

(21) L. Atkin, M. Feinstein and P. P. Gray, *Amer. Soc. Brewing Chemists Proc.*, p. 36 (1948).

(22) It was found that where a pair of components is frequently used, a simple nomogram may easily be constructed to give the volumes of liquids required as a function of density of the mixture. We have made extensive use of such nomograms. If the standards have been previously prepared, the time for a determination from preparation of the column to the recording of the unknown density is about 5 minutes. When aqueous standards and hydrophilic solids are used in a tube containing organic column components, it was found convenient to coat the column tubes with silicone in order to prevent the unknown and the calibration drops from sticking to the walls after accidental contact.

(23) E. M. Chamot and C. W. Mason, "Handbook of Chemical Microscopy," Vol. I, 2nd Edition, John Wiley and Sons, Inc., New York, N. Y., 1947, p. 145.

TABLE I

Substance	Density range of column	Crystalline state	Appearance in column	Density observed in gradient column	Literature value
		Linderstrøm-Lang gradient column[a]			
DL-Valine	1.15–1.35	Small fragments	Layered/2 mm.	1.32	1.316[19]
DL-Leucine	1.15–1.35	Thick flakes	Sharp layer	1.20	1.191[19]
		Modified gradient column with centrifugation[b]			
DL-Leucine	1.16–1.20	Thick flakes	Layered/3 mm.	1.192(1.189)	1.191[19]
DL-α-Amino-*n*-butyric acid	1.20–1.24	Flakes	Some distribution	1.230(1.221)	1.231[19]
DL-α-Alanine	1.35–1.45	Fine needles	Sharp layer	1.402	(1.40)[c](1.401)[d]
DL-Methionine	1.30–1.40	Very irregular fine flakes	Some distribution	1.340(1.310)	1.340[d,e,23]
Glycine[f]	1.48–1.65	Fine fragments	Sharp layer	1.606	1.607[19,g]
DL-Serine	1.48–1.65	Flakes	Layered/1 mm.	1.536(1.533)	1.537[d,e]
L-Valine	1.20–1.30	Flakes	Some distribution	1.232(1.22)	1.230[19]
Claviformin[f]	1.50–1.60	Large irregular fragments	Layered/2 mm.	1.535(1.528)	1.528 ± 0.003[10]

[a] Bromobenzene and xylene were the liquids used in preparing this column. [b] Bromobenzene and kerosene were the liquids used in preparing this column. The range of most of these columns was about 0.1 g./ml.; this difference spread over 5 cm., leads to a gradient of about 0.002 g./ml./mm. Positions were estimated to 0.5 mm., and the densities are, therefore, significant to about ±0.001 g./ml. Where the crystal layer was markedly distributed in the column after centrifugation, the low density limit of the distribution is indicated in parentheses. The true value of the density was taken as that of the sharp lower layer. [c] H. A. Levy and R. B. Corey, THIS JOURNAL, **63**, 2095 (1941). [d] Calculated from unit cell data of (*c*). [e] G. Albrecht and M. S. Dunn, unpublished data; "Handbook of Chem. and Physics," Chem. Rubber Publishing Co., 1947, p. 1438. [f] Bromobenzene and tetrabromoethane were used in preparing the columns for these two compounds. [g] T. Curtius, *J. prakt. Chem.*, **26**, 158 (1882).

Sharp layering can generally be obtained in the centrifuged gradient tube when care is taken in preparing the slurry. The modified gradient column procedure should be applicable to the rapid detection and separation of crystalline phases of different density.[24] A few exploratory experiments with some organic compounds have been made to investigate the range and sensitivity of the method. The results are briefly reported below.

Experimental

The modified gradient column employing 10-cc. test-tubes with centrifugation was used throughout. The materials were, with the exception of three antibiotic preparations, mixtures prepared from pure crystalline components by shaking or grinding together. The pure components were also recrystallized together from a common solvent. This was done to determine whether the compounds chosen formed molecular complexes or mixed crystals. The densities of the pure components used were determined, or the literature values remeasured before the mixtures were made up. Three pairs of compounds were chosen: (1) benzoic and *p*-toluic acid, (2) α- and β-naphthoic acid and (3) leucine and methionine.

The two pairs of compounds, benzoic and *p*-toluic acid, and α- and β-naphthoic acid, have been separated by L. C. Craig[25] by the countercurrent distribution method. A completely different approach to the separation of these compounds appeared of interest; this especially as the α- and β-naphthoic acids might be expected to have closely similar densities.

Commercial leucine separated by the usual procedure may be contaminated with methionine.[26] Weighed mixtures of these substances were prepared. Two to ten mg. of a preparation was used in each column determination.

Column Components.—Bromobenzene–kerosene or bromobenzene–tetrabromoethane columns were used for the amino acids and antibiotics density determinations. Aqueous columns (potassium phosphate and phosphoric acid) were used for the benzoic and *p*-toluic acid and α- and β-naphthoic acid determinations. Relatively insensitive gradient columns were used to determine the density range and sensitivity most appropriate to the study of the particular preparation. In order to obtain a more sensitive column area, it is necessary only to stir the existing column more thoroughly in the region of interest. This produces a non-linear gradient, which may, however, be easily calibrated by means of the micro Westphal balance technique.[20]

Results

Benzoic Acid and *p*-Toluic Acid.—The pure substances were observed to have the following densities: benzoic acid, $\rho = 1.332 \pm 0.003$[27]; *p*-toluic acid, $\rho = 1.272 \pm 0.004$ (observed, no literature value found). Mechanical mixtures and recrystallized mixtures both gave two layers at the same densities as the pure compounds. The benzoic acid dissolved in the potassium phosphate columns used initially, although even here the layers could be distinguished after centrifugation. Potassium phosphate columns saturated with benzoic acid, and phosphoric acid columns, were both used successfully to prevent solution.

α-Naphthoic Acid and β-Naphthoic Acid.—The densities of the pure compounds both before and after recrystallization from 50% aqueous ethanol were α-naphthoic acid, $\rho = 1.400 \pm 0.004$ (observed, no literature value found), β-naphthoic acid, $\rho = 1.352 \pm 0.003$.[29] A carefully weighed 1:1 mixture of these compounds recrystallized from 50% aqueous ethanol gave a single layer of density 1.365 ± 0.002. A recrystallized mixture of ratio 4α to 1β gave an α-layer (1.395) and the intermediate layer (1.365). A recrystallized mixture of a ratio 1α to 4β gave the intermediate layer (1.365) and a β-layer (1.342). The intermediate density appears to correspond to a true mixed crystal containing equal amounts of the α- and β-acids. The material has not been analyzed.

L-Leucine and L-Methionine.—The observed densities of the pure compounds both before and after recrystallization from water were: L-leucine, $\rho = 1.165 \pm 0.003$[19]; L-methionine, $\rho = 1.292 \pm 0.006$ (observed, no literature value found). Mechanical mixtures containing 10 and 1% of methionine were prepared. These separated readily in the columns; the 1% methionine layer was quite visible. Two mixtures were recrystallized from water: one containing a high percentage of L-methionine, the other a high percentage of L-leucine. Both these preparations gave sharp single layers with densities intermediate between that of

(24) The non-centrifuged gradient tube has been used by Boyer, Spencer and Wiley[7] for the investigation of the heterogeneity of dried copolymers.

(25) L. C. Craig, *J. Biol. Chem.*, **155**, 519 (1944).

(26) J. H. Mueller, *Science*, **81**, 50 (1935).

(27) This value agrees reasonably well with that calculated ($\rho = 1.334$) from the crystallographic unit cell dimensions.[28] The value of 1.2659 in the International Critical Tables appears to be in error.

(28) W. H. Bragg, *Trans. Chem. Soc.*, **121**, 2766 (1922).

(29) The density 1.077 is recorded for β-naphthoic acid in the International Critical Tables. This does not agree with the density observed by us for material crystallized from aqueous alcohol.

the two pure compounds, *i.e.*, high-methionine mixture, ρ = 1.260, high-leucine mixture, ρ = 1.179. The pure methionine showed rather poor layering, a characteristic noted for both the L and DL preparations. Some imperfection in the crystals must account for this density distribution (*e.g.*, occluded air or mother liquor).

Aureomycin Hydrochloride.—The aureomycin preparation used was Lederle tablets known to contain mannitol as excipient. The tablets after grinding gave a diffuse layer between 1.48 and 1.51 g./ml. with a concentration of material at 1.49 g./ml. A sample of mannitol (Eastman Kodak 155) recrystallized from ethanol gave a sharp layer at 1.489 ± 0.002 g./ml.[30] One hundred mg. of the preparation dissolved in 20 cc. of hot ethanol gave white crystals layering sharply at 1.500 ± 0.002. The remaining yellow residue still layered diffusely between 1.51 and 1.48. No further separation was attempted. No sample of a pure aureomycin was available for comparison.

Gramicidin.—The gramicidin preparation was made up of "mixed" crystals containing several active fractions.[31] This preparation gave a sharp heavy layer (ρ = 1.216 ± 0.004) with a small amount of widely distributed lighter material. The distribution was extremely stable; it remained essentially unchanged for several days. Where air bubbles are the cause of improper layering, the distributed material usually shifts rapidly as the air bubbles either grow or dissolve. It appears here that the distribution corresponds to compounds of differing densities in the original preparation.

Tyrocidin.—The tyrocidin preparation was made up of "mixed" crystals containing several active fractions.[31] The preparation layered out between 1.260 ± 0.002 and 1.285 ± 0.002. Both top and bottom cut offs were very sharp. This is a wider distribution than might be expected for a pure substance.

Conclusions.—The materials studied demonstrate some of the limitations as well as some of the possibilities of the method. The benzoic and *p*-toluic acids appear typical of the behavior of materials which do not affect each other upon recrystallization. They are easily and quantitatively separated. Where a mixed crystal of definite composition is formed upon crystallization (*e.g.*, α- and β-naphthoic acids), the method cannot be used as a true fractionation procedure. It may serve to separate an excess of one component, and, where the original materials have both been previously characterized, it will indicate the presence of a complex. In the case of leucine–methionine mixtures where a mixed crystal of varying composition appears to form, the method breaks down completely as a separatory tool. If the additivity of specific volumes is assumed for the mixture of density 1.179, this would correspond to leucine contaminated with approximately 10% methionine. Thus, the method may still be used to demonstrate contamination. None of the antibiotics investigated has been clearly fractionated in this study. The presence of mannitol in the aureomycin preparation has been demonstrated. The experiments suggest the possible usefulness of the gradient tube technique when associated with fractional recrystallization procedures. The phenomena observed with gramicidin and tyrocidin serve to emphasize the close similarity between the components of antibiotic mixtures. The dense material in the gramicidin study may correspond to some limited fractionation. The method is thus inapplicable where the compounds to be separated are molecules with density values within a very narrow range.

(30) Value recorded in International Critical Tables, ρ = 1.489.

(31) C. P. Hegarty, Sharp and Dohme, Inc., Glenolden, Pa., personal communication.

Use of the Gradient Column for Protein Crystal Density Determination

All protein crystals contain water of crystallization. In "wet"[32] crystals in equilibrium with their mother liquor, the amount varies within a wide range according to the protein.

When the crystals are removed from their mother liquor, they lose some water of crystallization and are termed "air-dried." The exact amount lost depends upon the relative humidity. If the crystals are put into aqueous media differing from that of their mother liquor, their composition is adjusted either by a loss of or a taking-up of water and/or other solute components.

The flotation method of density determination for protein crystals was developed by Adair and Adair.[33] It provides an accurate technique for the measurement of protein crystal density in media of known composition. Since the densities of protein crystals vary with the nature of the suspending medium, these measurements have limited significance for wet crystal density determination. These workers used a series of media differing by 0.001 density unit in a set of centrifuge tubes. Crystals were put into the tubes in turn and centrifuged. By this method, the range from crystalline suspension at the surface to deposition was covered. Centrifugation of small crystals took 5 to 45 minutes and was accompanied by an increase of 1 to 3° in temperature. The authors suggested the use where possible of large crystals in thermostated tubes without centrifugation.

X-Ray measurements of the unit cell are commonly made both on the wet crystal immersed in its mother liquor and also on air-dried crystal. In order to correlate X-ray and density measurements for the determination of the molecular weight of the asymmetric unit, the density measurements must be made on true wet and dry crystals.

The precision of the determination of protein crystal densities by the flotation method in certain solvents has been reported as of the order of 1 in 400 or higher.[34,35] The uncertainty in the absolute value of the densities thus obtained as related to the wet crystal unit cell is probably of the order of 2–4%. Larger absolute errors than this have been recorded for wet protein crystals because of the unsuspected effect of the medium.[35]

In recent studies McMeekin, *et al.*,[36,37] have used the flotation technique with bromobenzene–xylene mixtures for the determination accurate to 0.5% of the absolute density of large wet crystals of β-lactoglobulin. These measurements are certainly the most accurate yet recorded. Their accuracy

(32) By "wet" crystals, we shall mean specifically crystals with the unit cell volume and composition of crystals in equilibrium with their mother liquor.

(33) G. S. Adair and M. E. Adair, *Proc. Roy. Soc.* (*London*), **B120**, 422 (1936).

(34) (a) Density of Tobacco Seed Globulin, D. M. Crowfoot and I. Fankuchen, *J. Gen. Physiol.*, **24**, 315 (1941); (b) Density of Ribonuclease, I. Fankuchen, *ibid.*, **24**, 315 (1941).

(35) D. M. Crowfoot and D. P. Riley, *Nature*, **141**, 521 (1938). The density of 1.257 for β-lactoglobulin recorded here is approximately 10% greater than the true wet crystal density of 1.146.[36]

(36) T. L. McMeekin and R. C. Warner, THIS JOURNAL, **64**, 2393 (1942).

(37) T. L. McMeekin, M. L. Groves and N. J. Hipp, *ibid.*, **72**, 3662 (1950).

TABLE II

AIR-DRIED PROTEIN CRYSTAL DENSITIES

Substance	Density range of column	Crystalline state	Appearance in column	Density observed in gradient column	Literature value
Air-dried insulin	1.15–1.35[a]	Powder	Sharp layer	1.31(1.300–1.31)	1.315[d,e]
Air-dried insulin	1.28–1.32[b]	Powder	Sharp layer	1.312(1.302–1.312)	1.315[d,e]
Air-dried β-lactoglobulin	1.24–1.34[c]	Large single crystals		1.257(±0.001)	1.260[36]
Xylene-dried β-lactoglobulin	1.24–1.34[a,c]	Large single crystals		1.254(±0.001)	1.260[36]

[a] Measured in 20-cc. non-centrifuged gradient column. [b] Measured in 10-cc. centrifuged gradient column. [c] Measured in 10-cc. non-centrifuged gradient column. [d] D. M. Crowfoot, *Nature*, **135**, 591 (1935). [e] The zinc content of insulin crystals may vary considerably and this affects their density (*f*). The good agreement recorded between observed and literature values may have been affected by this discrepancy. [f] E. J. Cohn, J. D. Ferry, J. J. Livingood and M. H. Blanchard, THIS JOURNAL, **63**, 17 (1941).

is dependent upon the relative ease with which the large crystals may be rapidly freed from adhering mother liquor and immersed in a liquid medium.

The gradient column method appeared applicable to the accurate measurement of the density of both large wet protein crystals and all types of air-dried protein crystals. It is somewhat more rapid and requires less material than the flotation method. Instead of the relatively large number of prolonged centrifugations required by the flotation method, only a single run using one or two crystals is required in the gradient column.

In order to investigate the usefulness of this technique, measurements have been made of the density of wet and dry β-lactoglobulin crystals and air-dried insulin crystals.

Experimental

The liquids used by McMeekin and Warner were bromobenzene and xylene saturated with water to prevent equilibration between wet crystals and the immersion media during the experiment. We have set up gradient columns using these two components both air-dried (in equilibrium with the air at room temperature), and also saturated with water. In measuring the density of large protein crystals centrifugation proved unnecessary. The columns were set up in 10-cc. tubes as a standard practice. The densities of wet crystals of β-lactoglobulin of various sizes were measured. The time taken to wipe the crystal dry was recorded.

The technique used for the dry crystal densities was not different from that used for the measurement of ordinary non-protein crystals, except that both column components were air-dried to prevent exchange of water between liquids and crystals.

Results

Crystals of β-lactoglobulin were measured in bromobenzene–kerosene columns at 24° without centrifugation. The density range was 1.10–1.20. Two columns were set up, one using air-dried components in equilibrium with the air at room temperature, the other with components saturated by shaking with 0.15 molar salt solution. The approximate crystal weight was calculated from the dry weight measured after the experiment. The drying times were measured from the moment the crystal was removed from its mother liquor until it was dropped after wiping dry into the gradient tube. The density was measured after 5 minutes, a standard period adopted for all crystals. The large crystals reached an equilibrium position in about 3 minutes. Smaller crystals took up to 5 minutes. After 10 minutes, all the crystals increased slowly in density due to a loss of water to the column components.

Seven crystals of various weights were used for the determination in each column. The differences between saturated and dry columns were always within the limits of experimental error. The minimum density obtained for wet β-lactoglobulin crystals was 1.144 ± 0.002. This figure compares with the value obtained by McMeekin and Warner[36] of 1.146.[38] This lowest figure was obtained with the largest crystal used, and with drying times of approximately 25 seconds. Comparing crystals of different sizes (*e.g.*, weighing 2.0–0.2 mg., respectively), it was evident that the larger the crystal used, the more accurate the measurement. This is clearly due to the greater surface area for water loss per unit weight of the smaller crystal. When we compared the measurements on two crystals of approximately the same weight (2.0 mg.), dried during different lengths of time (20 and 60 seconds), it was evident that a slight increase in the drying time markedly affects the observed density.

Calculations made, using McMeekin and Warner's data on the rate of drying, suggest that a crystal of β-lactoglobulin, of the common habit, weighing 10 mg. will show an approximate density increase of 0.002 g./ml. per minute of exposure to the air. Crystals weighing 1.0 mg. will show density increments of 0.005 g./ml. per minute, and those weighing 0.1 mg. will show twice this density increase.

The observations are approximately in agreement with these calculations. Both observation and calculation provide evidence that this method, like the flotation technique, gives accurate values only for large wet crystals where the water loss on limited exposure to air is negligible. Small crystals cannot be easily wiped dry. Whilst both this method and the flotation technique are equally limited in applicability, both appear equally sensitive and the gradient column method has the advantage of marked rapidity. It also requires less material.

In Table II, the results obtained on two air-dried proteins are recorded. The insulin crystal dry density was measured in both non-centrifuged and centrifuged gradient columns.

The bulk of the dry insulin powder was layered at 1.31. There was some limited distribution of crystals over the density range shown in parentheses. The limits of accuracy given for the β-lactoglobulin crystals describe the error in the measurement of the position of the large crystals used. The air-dried density for insulin agrees with the value recorded in the literature. The differences between the densities of air-dried and xylene-dried β-lactoglobulin crystals may be real. These densities were measured at the same time in the same gradient column. Small differences in density gain significance when the crystals may be clearly seen suspended at different heights in a column.

Discussion

The gradient tube technique appears to provide a rapid and accurate method for the determination of crystal densities. For a pure phase, the method, carefully applied, can yield as accurate a value for the density as that of any of the standard procedures now in use. It has the added advantage of indicating automatically any density distribution due

(38) The density found by us does not agree well with the latest value obtained by McMeekin and his associates[37] of 1.153 ± 0.002. A preparation of β-lactoglobulin crystals grown by Dr. T. L. McMeekin has, therefore, been examined in his laboratory (private communication) and also by us. Both groups of workers report a density of 1.148–1.150. It appears from this that differences of density from preparation to preparation of β-lactoglobulin crystals are real, though as yet unexplained.

to crystalline imperfections which in other methods are averaged in the result. The emphasis in this study has been upon a technique for the rapid examination of small quantities of material with respect to the density of the solid phases present. For the purposes of rapid survey, measurements to an accuracy of $\pm 0.2\%$ can be made in 5 minutes. The use of the centrifuge is largely responsible for this rapidity.

The limitations of the gradient column technique in the study of crystalline homogeneity have been discussed. Where it is applicable, the method appears to have several advantages. No severe changes in environment which might affect labile compounds are involved. Materials once crystallized need not be dissolved. The recovery, with care, may be made quantitative. It has been shown that a 1% impurity may be easily observed. Further investigations on the sensitivity have still to be made. The sensitivity has been limited in this study to the detection of a mass of crystalline fragments large enough to be visible to the naked eye. The microscopic examination of a microgradient tube should lead to the observation of a single very small crystal.

The sensitivity of the density determination as an indication of the purity and identity of a crystalline phase appears to have been neglected. Since the optically active and racemic forms of a compound almost invariably have different crystal densities, this method offers a rapid quantitative procedure for distinguishing between these two forms. Application of the method may establish the presence of a complex or a mixed crystal rather than a pure compound of known density.

Wet and dry protein crystal densities have been studied by the gradient column technique. This method provides a non-equilibrium procedure comparable in accuracy to, and more rapid than the flotation technique hitherto used. Small differences in density between crystals subjected to different media or drying procedures are visibly demonstrated by the use of a single gradient column for their comparison. The results demonstrate the speed with which protein crystals change in composition upon exposure to air and emphasize the value of an equilibrium method for their study. Non-equilibrium methods are only applicable to the study of large crystals. An equilibrium method for the study of smaller protein crystal densities has, therefore, been developed. It will be described elsewhere.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, BROOKHAVEN NATIONAL LABORATORY]

The Mass Spectra of Some Deuterated Isopropyl Alcohols[1]

BY LEWIS FRIEDMAN AND JOHN TURKEVICH

The mass spectra of $(CH_3)_2CHOH$, $(CH_3)_2CHOD$, $(CH_3)_2CDOH$ and $(CH_3)_2CDOD$ are reported and the mechanism of the formation of the more abundant fragment ions is discussed. The results support the concept of localized charge on the oxygen atom in the more abundant ions.

Introduction

The mass spectra of $(CH_3)_2CHOH$, $(CH_3)_2CHOD$ and $(CH_3)_3CDOD$ were needed in connection with studies on the mechanism of the catalytic reduction of acetone with deuterium. The mass spectrum of isopropyl alcohol has been previously reported[2] but no data are available on the mass spectra of deuterium substituted alcohols.[3]

Experimental

Isopropyl alcohol was prepared by hydrolysis with ordinary water of aluminum isopropoxide which had been previously purified by distillation. Isopropyl alcohol O–D was prepared by hydrolysis of the same compound with 99.8% D_2O. The absence of an O–H absorption band at 2.95 μ in the infrared absorption spectrum of the isopropyl alcohol O–D indicates less than 5% impurity of $(CH_3)_2CHOH$. Isopropyl alcohol 2-C–D–O–D was prepared by catalytic reduction of acetone with deuterium.[4] Isopropyl alcohol 2-C–D–OH was prepared by hydrolysis of the 2-C–D–OD with light water.

The isotopic content of these preparations was determined in the following way from the mass spectrometer data. The relative intensity of the parent ion is small and cannot be used as a criterion of purity under controlled electron bombardment. However in the mass region 45–48, the most intense ion corresponds to a fragment produced by the loss of a methyl group. In the case of the dideutero compound this ion has the mass 47 and its relative intensity is set at 100 units. Isopropyl alchol containing more than two D atoms may be estimated from the intensities of ions above mass 47 in this region of the spectrum. Ions of mass 48 are present to the extent of 3.64 units of which approximately 2.2 are ascribed to natural C^{13}. The remaining 1.4 units arise from the dissociation of a trideutero isopropyl alcohol formed by exchange in the reduction process. Since the probability of losing the CH_3 or CH_2D methyl group is approximately the same, the residual peak intensity of 1.4 units at mass 48 indicates an upper limit of 3% for trideutero isopropyl alcohol. The concentration of monodeutero isopropyl alcohol may be estimated from the relative intensity of ions of mass 46. In ordinary isopropyl alcohol the corresponding process, *i.e.*, the loss of CH_4 or $CH_3 + H$, gives rise to a peak of 4.3 units at mass 44. If we subtract 4.3 units for this process which normally occurs from the observed 23.7 units at mass 46 in the dideutero isopropyl alcohol spectrum, we obtain 19.4 units which may be due to mono-

(1) Research carried out under the auspices of the U. S. Atomic Energy Commission. Presented before the Division of Physical and Inorganic Chemistry at the 116th Meeting of the ACS, September, 1949.

(2) (a) American Petroleum Institute, Research Project 44, National Bureau of Standards 76, 285; (b) B. W. Thomas and W. D. Seyfried, *Anal. Chem.*, **21**, 1023 (1949); (c) A. P. Gifford, S. M. Rock and W. J. Comaford, *ibid.*, **21**, 1027 (1949).

(3) Since this work has been completed a paper by F. E. Condon, H. L. McMurry and V. Thornton, *J. Chem. Phys.*, **19**, 1010 (1951), has been published containing the mass spectra of $(CH_3)_2CDOH$.

(4) L. Friedman and J. Turkevich, THIS JOURNAL, **74**, 1669 (1952).